Host cadavers protect entomopathogenic nematodes during freezing

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Abstract

The entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, *Steinernema glaseri*, and *Steinernema feltiae* were exposed to freezing while inside their hosts. Survival was assessed by observing live and dead nematodes inside cadavers and by counting the infective juveniles (IJs) that emerged after freezing. We (1) measured the effects of 24 h of freezing at different times throughout the course of an infection, (2) determined the duration of freezing entomopathogenic nematodes could survive, (3) determined species differences in freezing survival. Highest stage-specific survival was IJs for *S. carpocapsae*, and adults for *H. bacteriophora*. When cadavers were frozen two or three days after infection, few IJs emerged from them. Freezing between five and seven days after infection had no negative effect on IJ production. No decrease in IJ production was measured for *H. bacteriophora* after freezing. *H. bacteriophora* also showed improved survival inside versus outside their host when exposed to freezing.
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1. Introduction

Entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) are currently used in several agricultural production systems for insect control with varying degrees of success (Shapiro-Ilan et al., 2002). Infective third-stage juvenile (IJ) nematodes are the only free-living stage. They search the soil habitat for a suitable host. During this stage, the nematodes do not feed or develop. Upon entering an insect host through natural openings, the nematodes release their symbiotic bacteria (*Xenorhabdus* spp. for *Steinernema* spp. and *Photorhabdus* spp. for *Heterorhabditis* spp. (Boemare, 2002)) which, in combination with toxins produced by the nematodes kill the host within three days. The bacteria and degrading host tissues provide the nutrient source for the developing nematodes. The nematodes usually go through two generations inside the host in a period of about 10 days, depending upon temperature and the initial inoculation density, after which another generation of IJs emerges from the host (Adams and Nguyen, 2002).

Despite the widespread use of entomopathogenic nematodes little is known of their survival ecology in soil; whether they were applied to an area or were endemic. Generally, persistence of applied entomopathogenic nematodes is short (e.g., 2–3 weeks) (Duncan and McCoy, 1996; McCoy et al., 2000). Yet on rare occasions entomopathogenic nematodes have been reported to persist for a number of years or seasons (Klein and Georgis, 1992; Parkman et al., 1993a,b). Their survival as formulated commercial product is also poorly understood and is one of the important limitations to the success of these biological control agents on the market. Temperature extremes are one of the factors that limit entomopathogenic nematode survival (Glazer, 2002).

A wide range of studies has been conducted on entomopathogenic nematode IJ responses to warm and cold temperatures, in terms of survival and infectivity. One isolate of *Heterorhabditis bacteriophora* Poinar (IS5 strain) from Israel has shown high levels of heat tolerance by surviving at 40 °C and infecting and reproducing in hosts at 30 °C (Shapiro et al., 1996). The interest in having entomopathogenic nematode IJs survive warmer

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than normal temperatures has also led to the development of a transgenic nematode with the heat-inducible hsp-70 gene from *Caenorhabditis elegans* that increases IJ survival at 40 °C. (Hashmi et al., 1998). Tolerance to cold temperatures has also been sought in various ways. Brown and Gaugler (1998) showed that *Steinernema carpocapsae* (Weiser), *Steinernema glaseri* (Steiner), and *Steinernema riobravis* Cabanillas, Poinar, and Raulston IJs were freeze-tolerant at −4 °C. *S. carpocapsae* had the highest survival and *S. glaseri* the lowest.

Despite several studies to determine IJ survival in extreme temperatures, a basic aspect of their ecology that remains enigmatic is how entomopathogenic nematodes survive adverse conditions in nature, and in particular how species in temperate climates survive freezing winter temperatures. The role of the cadaver in the biology of entomopathogenic nematodes has been examined in terms of infectivity (Shapiro and Lewis, 1999) and dispersal (Shapiro and Glazer, 1996). Here, we compared the freezing survival of four species of entomopathogenic nematodes within their host cadavers to IJ nematodes frozen in sand. Our aim was to measure reproductive success after freezing for entomopathogenic nematodes at various temperatures and to determine which life stages (if any) within the host were able to withstand freezing temperatures. This information is important for two reasons. First, it may shed light on a way that entomopathogenic nematodes naturally endure prolonged exposure to freezing temperatures. Second, this information may facilitate development of methods to store entomopathogenic nematodes in their infected hosts. The nematodes or the cadavers themselves could then be applied for pest suppression.

2. Materials and methods

2.1. Culture maintenance

All entomopathogenic nematode cultures were maintained in last instar greater wax moth larvae, *Galleria mellonella* (L.), according to procedures described in Woodring and Kaya (1988). Nematodes were maintained at approximately 22 ± 3 °C. Entomopathogenic nematode species tested were: *S. carpocapsae* (All strain), *Steinernema feltiae* (Filipjev) (SN strain), *S. glaseri* (NC strain) and *H. bacteriophora* (HP88 strain). All of these cultures have been maintained under similar conditions for at least five years, and have not been purposefully subjected to any kind of selection for freezing or cold tolerance.

2.2. Stage-specific survival

Experiment 1 was designed to determine which developmental stage(s) of *S. carpocapsae* and *H. bacte-*

riophora, if any, survived freezing temperatures within the host cadaver and eventually produced IJs. On Day 0, *G. mellonella* larvae were exposed individually to 25 IJs of *S. carpocapsae* or 50 IJs of *H. bacteriophora* in 60 mm petri dishes lined with filter paper. We used different levels of exposure because *S. carpocapsae* is more efficient infecting hosts in a petri dish arena than *H. bacteriophora*. Cadavers were subjected to −1, or −8 °C for a period of 24 h in the following procedure. Twenty-four hours after infection (or on "Day 1"), 10 larvae contained in a 60 mm petri dish with dry filter paper were placed into a freezer at one of the above-mentioned temperatures for 24 h, then removed and set up in a White trap (White, 1927) at approximately 22 ± 3 °C. This procedure was followed daily, until IJs began to emerge from cadavers before the freezing treatment could start. So, 10 cadavers were exposed to 24 h of freezing temperatures 1, 2, 3, 4, etc. days after infection. In other words, the sample size is 10 for each treatment. A treatment is a day-by-temperature combination. The total number of IJs that emerged was either counted or estimated by dilution and recorded from each cadaver. Data were analyzed by analysis of variance and means were compared with the Student–Newman–Keules test.

We also dissected five infected larvae from each temperature/day combination for each test and determined which stages of nematodes were alive, if any, and what proportion of the nematodes in the cadaver were alive. A maximum of 50 nematodes were checked determined to be dead or alive, and their stage was recorded. The proportion of live nematodes was compared among treatments by analysis of variance, with day as the response variable and means were compared with the Student–Newman–Keules test. Data were transformed by $\sqrt{\text{arcsin}}$ before analysis.

2.3. Duration of freezing

Experiment 2 examined the effects of different durations of exposure of cadavers to freezing temperatures on IJ production. *G. mellonella* larvae were infected as above with *H. bacteriophora*, *S. carpocapsae*, *S. feltiae* (50 IJs per host), or *S. glaseri* (50 IJs per host). Cadavers were exposed to freezing temperatures 7 days after the initial exposure, which corresponded with a post-infection duration where *S. carpocapsae* and *H. bacteriophora* reproduction (in previously frozen cadavers) and survival were recorded for most frozen cadavers in Experiment 1 (see Figs. 1–4).

The treatments were conducted as follows: *S. carpocapsae* was tested at −15 °C, *S. feltiae* was tested at −15 and −8 °C, *H. bacteriophora* was tested at −15 and −8 °C, and *S. glaseri* was tested at −15 and −8 °C. The duration treatments consisted of cadavers that were exposed to freezing temperatures for 0, 24, 48, 72, or 168 h. Ten cadavers were exposed to each temperature/
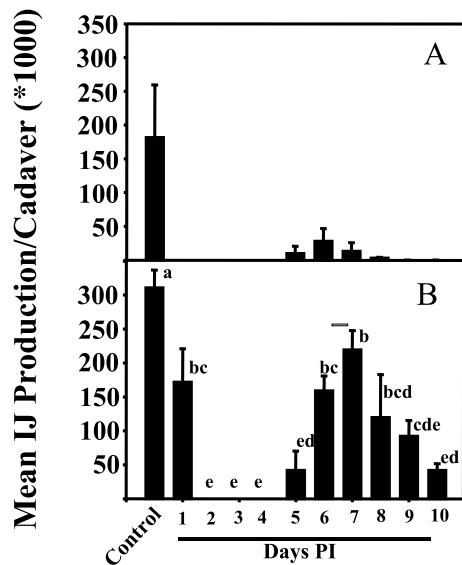
Fig. 1. Mean infective juvenile production (±SEM) from *Heterorhabditis bacteriophora*-infected cadavers subjected to −1 °C for 24 h at various times after infection. The experiment was conducted twice, and each figure depicts the data collected from one experiment. Bars with different letters are significantly different (α = 0.05) within each experiment.
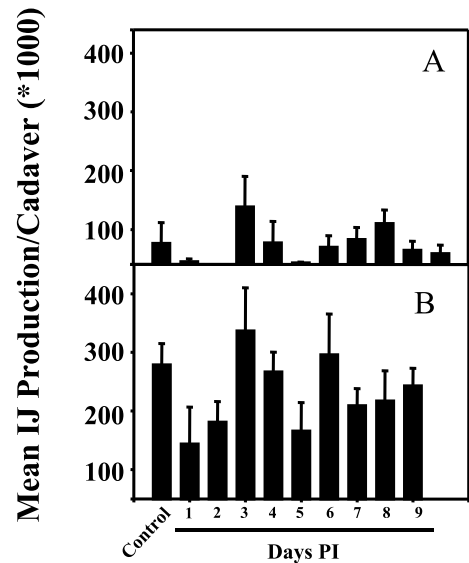

Fig. 3. Mean infective juvenile production (±SEM) from *Steinernema carpocapsae*-infected cadavers subjected to −1 °C for 24 h at various times after infection. Each figure depicts the data collected for one experiment. Bars with different letters are significantly different (α = 0.05) within each experiment.
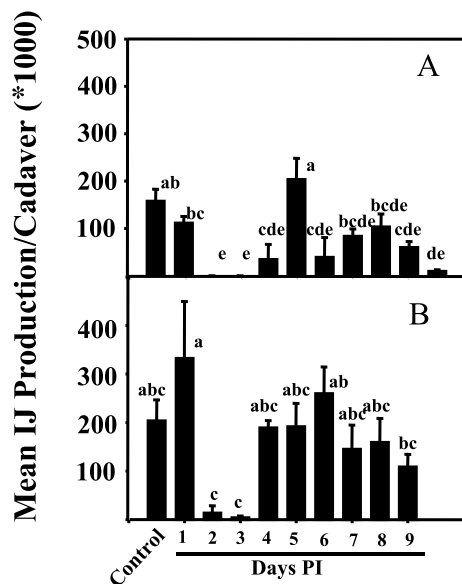

Fig. 2. Mean infective juvenile production (±SEM) from *Heterorhabditis bacteriophora*-infected cadavers subjected to −8 °C for 24 h at various times after infection. The experiment was conducted twice, and each figure depicts the data collected from one experiment. Bars with different letters are significantly different (α = 0.05) within each experiment.
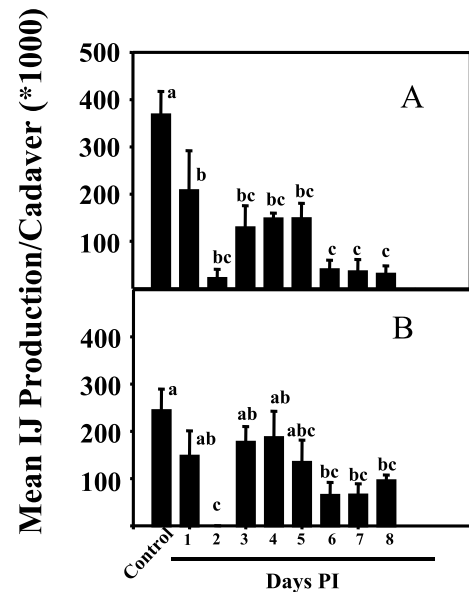

Fig. 4. Mean infective juvenile production (±SEM) from *Steinernema carpocapsae*-infected cadavers subjected to −8 °C for 24 h at various times after infection. Each figure depicts the data collected for one experiment. Bars with different letters are significantly different (α = 0.05) within each experiment.

duration treatment. After the assigned time period, cadavers were placed individually into White traps at ambient laboratory temperature (ca. 22–25 °C). The total number of IJs produced per cadaver was either counted or estimated by dilution and recorded.

The mean numbers of entomopathogenic nematode IJs were compared among treatments within species.

Data were analyzed by analysis of variance. Means were compared by the Student–Newman–Keules test.

2.4. Freezing infective juveniles in sand

The final test compared the survival of IJs in moist sand at −8 °C. Sand was mixed to a uniform moisture of

10% w/w. Infective juveniles of *S. carpocapsae*, *S. glaseri*, *S. feltiae*, or *H. bacteriophora* were collected from White traps and 2500 IJs were released into each of 30 60 mm petri dishes filled with the sand and immediately placed into the freezer. After one, two, or seven days 10 petri dishes were removed from the freezer and % IJ survival was estimated by counting all nematodes in a sub-sample of the sand and determining how many were alive by probing. Data were transformed by $\sqrt{\arcsin}$ and analyzed by analysis of variance. Means were compared with the Student–Newman–Keules test.

3. Results

3.1. Stage-specific survival

The level of reproduction from cadavers frozen at different times in the infection process varied significantly with the day of freezing for both species tested. For *H. bacteriophora* at both temperatures, reproduction from cadavers tended to be lowest when frozen 2 to 3 days after infection, increased during days 5–8 and decreased again toward the end of the observation period (8–10 days). Infective juvenile production following freezing on day 1, 5, 6, or 7 after infection was significantly greater than at least some of the other days at either temperature. For *S. carpocapsae*, there were less consistent results. When frozen at −1 °C, there were no differences in IJ yield between any of the day treatments and the non-frozen control. The −8 °C treatment showed a pattern of IJ production more similar to that of both of the *H. bacteriophora* treatments; in one of the trials (Fig. 4B) cadavers frozen three days after infection produced fewer IJs than any of the other days. For *H. bacteriophora* and *S. carpocapsae*, IJ production by cadavers frozen on day 5–7 after infection was as high as the control for all tests except one at −1 °C (Figs. 1B and 4A, respectively).

Overall, survival in frozen cadavers, as determined by dissection, was similar to the reproduction results, but the two data sets do not correspond exactly (Table 1A and B). For example, cadavers with *H. bacteriophora*- frozen at −1 °C six and seven days after infection had the highest survival rate of the nematodes inside, which corresponds to days when reproduction was also high. At −8 °C, *H. bacteriophora* survival was highest on days seven, eight, and 10 after infection. Percent survival of nematodes inside cadavers with *S. carpocapsae* did not differ with day, which corresponds with reproduction data (Fig. 3). At −8 °C, *S. carpocapsae* survival was highest when frozen one day after infection. When the cadavers were dissected and the live nematodes were grouped into classes of IJs, non-dauer juveniles or adults, the results varied with species and temperature. All days' dissections were grouped to represent these data, and

Table 1
Percent daily survival of (A): *Heterorhabditis bacteriophora*, (B): *Steinernema carpocapsae* in cadavers subjected to two freezing temperatures

Temperature	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
(A)										
−1	5.6 ± 5.6[b]	0[b]	0[b]	4.8 ± 4.8[b]	7.4 ± 4.9[b]	22.4 ± 6.5[a]	21.6 ± 6.0[a]	5.3 ± 2.0[b]	2.5 ± 1.2[b]	0[b]
−8	11.1 ± 7.0[bc]	4.2 ± 4.2[bc]	0[c]	14.8 ± 5.9[abc]	3.4 ± 2.8[bc]	9.2 ± 3.7[bc]	29.8 ± 4.5[a]	13.8 ± 4.2[abc]	9.8 ± 3.1[bc]	16.9 ± 4.6[ab]
(B)										
−1	12.5 ± 6.1[a]	1.9 ± 1.4[a]	13.7 ± 5.1[a]	23.1 ± 8.5[a]	3.1 ± 2.5[a]	3.0 ± 1.1[a]	11.7 ± 4.1[a]	17.0 ± 4.9[a]	9.0 ± 2.7[a]	
−8	24.3 ± 4.9[a]	0[c]	3.3 ± 3.3[c]	0.3 ± 0.3[c]	1.4 ± 0.8[c]	2.0 ± 1.3[c]	3.7 ± 1.1[c]	10.6 ± 2.3[b]		

Means and SEMs are represented and numbers labeled with different letters are significantly different ($\alpha = 0.05$) within temperatures.

Table 2
Proportions of live nematodes classed into age groups as infective juveniles (IJ), non-dauer juveniles (NDJ) or adults found in dissected cadavers

Species	Temperature (°C)	Proportion of live nematodes		
		IJ	NDJ	Adult
H.B.	−1	0.46 ± 0.09^a	0.004 ± 0.004^b	0.53 ± 0.09^a
	−8	0.41 ± 0.07^a	0.20 ± 0.06^b	0.39 ± 0.06^a
S.C.	−1	0.51 ± 0.07^a	0.26 ± 0.06^b	0.26 ± 0.06^b
	−8	0.74 ± 0.08^a	0.22 ± 0.08^b	0.04 ± 0.03^b

H.B = *Heterorhabditis bacteriophora*, S.C. = *Steinernema carpocapsae*.
Means and SEMs are represented and numbers labeled with different letters are significantly different ($\alpha = 0.05$) within temperatures.

the results represent only cadavers where live nematodes were found. Infective juveniles made up the majority of survivors for *S. carpocapsae* at both temperatures, however for *H. bacteriophora*, at −1 °C, adults made up the majority of the live nematodes and at −8 °C, adults made up 41% of the live nematodes (Table 2).

3.2. Duration of freezing

The amount of time that a cadaver could be frozen and still produce IJs varied with temperature and species. All four species of entomopathogenic nematodes produced some IJs after being frozen at one of the temperatures tested (Figs. 5–9). At −15 °C, cadavers infected with *S. carpocapsae* or *S. feltiae* produced numbers of IJs significantly lower than non-frozen cadavers at all the freezing durations (Figs. 5 and 6). Cadavers infected by *H. bacteriophora* or *S. glaseri* produced no IJs when frozen at −15 °C (data not shown). Cadavers infected with *S. feltiae*, *S. glaseri*, or *H. bacteriophora* all produced some IJs when frozen at −8 °C (Figs. 7–9). For *S. feltiae* and *H. bacteriophora*-infected cadavers, IJ production was not significantly lower than that of unfrozen cadavers. *S. feltiae* had greater IJ production after 24 h of freezing than any duration longer and at 48 h, production was significantly

lower than at any other duration. *S. glaseri* did not produce any IJs after periods of freezing longer than 24 h.

3.3. Freezing infective juveniles in sand

Freezing IJs in sand yielded results that were varied among spp. (Fig. 10). *S. feltiae* IJs and *S. carpocapsae* IJs each had about 50% survival after 1 week of freezing
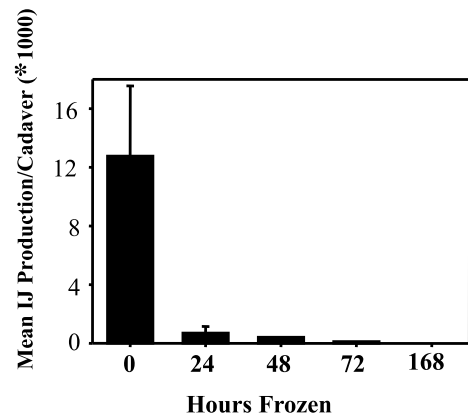

Fig. 6. Mean infective juvenile production (±SEM) from *Steinernema feltiae*-infected cadavers subjected to −15 °C for various durations. Bars with different letters are significantly different ($\alpha = 0.05$).
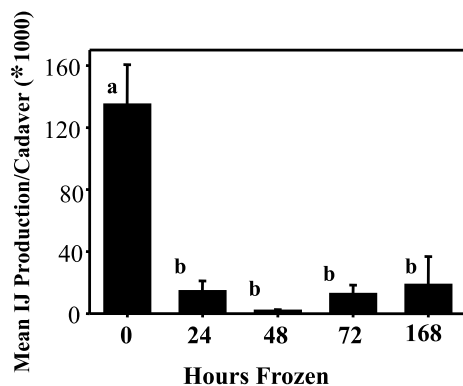

Fig. 5. Mean infective juvenile production (±SEM) from *Steinernema carpocapsae*-infected cadavers subjected to −15 °C for various durations. Bars with different letters are significantly different ($\alpha = 0.05$).
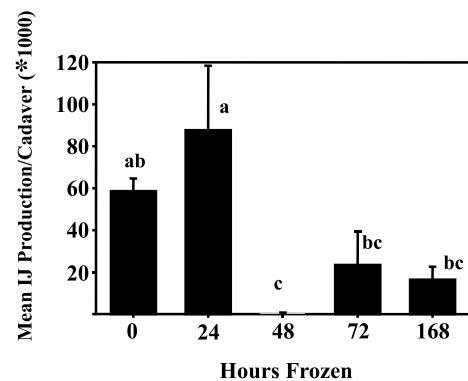

Fig. 7. Mean infective juvenile production (± SEM) from *Steinernema feltiae*-infected cadavers subjected to −8 °C for various durations. Bars with different letters are significantly different ($\alpha = 0.05$).
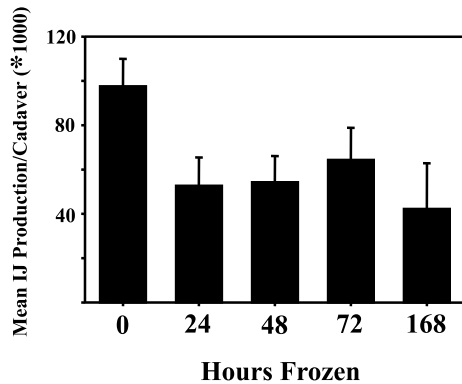
Fig. 8. Mean infective juvenile production (±SEM) from *Heterorhabditis bacteriophora*-infected cadavers subjected to −8 °C for various durations. Bars with different letters are significantly different (α = 0.05).
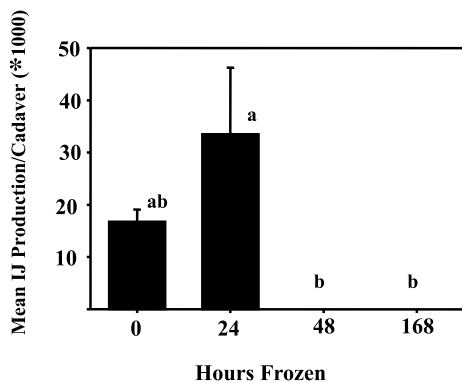

Fig. 9. Mean infective juvenile production (±SEM) from *Steinernema glaseri*-infected cadavers subjected to −8 °C for various durations. Bars with different letters are significantly different (α = 0.05).

at −8 °C, which represented a significant drop from survival after 1 day of freezing. Infective juvenile *S. glaseri* and *H. bacteriophora* suffered complete mortality after 1 week of freezing. Two days of freezing in these species resulted in a significant drop in viability compared to 1 day of freezing.

4. Discussion

Entomopathogenic nematodes survive freezing temperatures inside their host and go on to reproduce once moderate temperatures resume. The IJ stage of most entomopathogenic nematode species tested are freezing tolerant (Brown and Gaugler, 1996), which means that they freeze as ice nucleation penetrates the cuticle or body orifices as the surrounding water freezes (Wharton, 1995). The exception is *Heterorhabditis zealandica* which is freeze avoiding (Wharton and Surrey, 1994). Since we did not record whether the various life stages of nematodes inside the host cadaver actually froze or not, we cannot say why the nematodes inside the cadavers survived to reproduce. The proportion of nematodes that survived inside the host was relatively low, however, enough survived inside the host to maintain a viable infection and produce a new generation of IJs. Even taking into account the missing information, the fact that cadavers can be frozen and then thawed and still produce IJs is significant.

Our data suggest that nematodes do not tolerate freezing temperatures as well from 48–72 h after infection as at other times sooner or later after infection. All other studies of entomopathogenic nematodes have
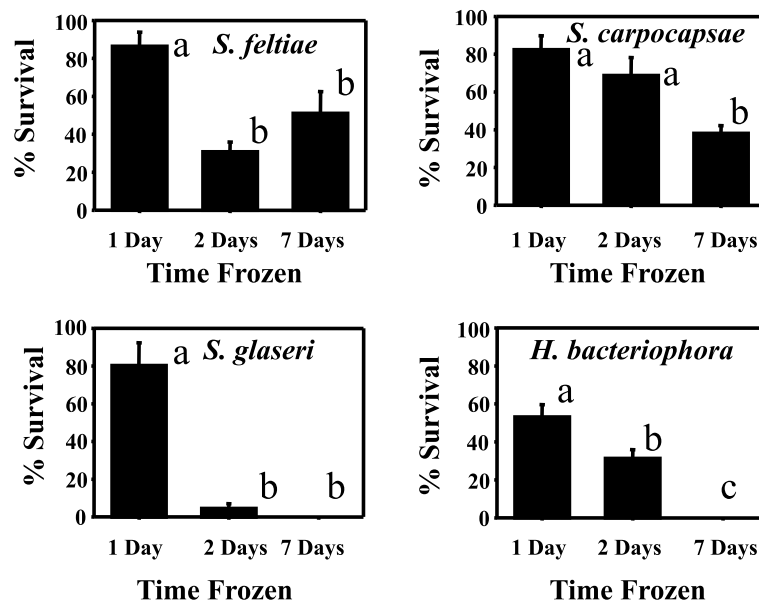

Fig. 10. Survival of *Steinernema feltiae*, *Steinernema glaseri*, *Steinernema carpocapsae*, and *Heterorhabditis bacteriophora* after storage in sand for various durations −15 °C. Bars with different letters are significantly different (α = 0.05).

targeted IJs, so there are no other data available on the freezing survival of parasitic stages of entomopathogenic nematodes. Tyrrell et al. (1994) studied the survival of the nematode *Wetanema* sp. in its insect host *Hemideina maori* (Orthoptera: Stenopelmatidae), an alpine weta native to New Zealand. The host and it's nematode parasites are both freezing tolerant. They went on to suggest that there may be a correlation between a nematode's size and its ability to survive freezing; inside the weta, the larger stages of parasitic nematodes survived at higher rates than did smaller ones and in the free-living antarctic nematode *Panagrolaimus davidi* similar survival patterns are apparent when frozen (Wharton and Ferns, 1995). Our data for *H. bacteriophora* may support this hypothesis in that the majority of live nematodes found in cadavers were adults. However, most surviving nematodes in the *S. carpocapsae* infections were IJs. In more natural infections, where not all IJs enter the host at the same time, mixed-stage populations of nematodes inside the host are likely the norm, and a decrease in the survival of a specific stage would probably go undetected because the other stages would survive to reproduce.

Infective juvenile production declined as cadavers were frozen for longer periods of time for all species tested except *H. bacteriophora*. We have no plausible explanation why *H. bacteriophora* responded differently to these treatments. Since we did not measure survival directly in the experiment where freezing duration was tested, it is difficult to compare our data with those of Brown and Gaugler (1996, 1998), who measured *Steinernema* spp. IJ survival and infectivity after freezing and storage. However, one similarity did occur between the studies; they found that *S. glaseri* IJs did not fare well after freezing and we found that *S. glaseri*-infected cadavers produced no IJs after 48 h of freezing whereas all the other species tested did. Grewal et al. (1994) suggested that, based upon temperature preference, *S. glaseri* was of tropical origin and ill-suited to cold temperature survival.

The mechanism of survival for entomopathogenic nematodes inside their cadavers is not clear from our study, however we can make some speculations. Brown and Gaugler (1998) found that freezing nematode IJs in 20% glycerol instead of water increased their survival significantly. This was not surprising because glycerol is commonly used as a cryoprotectant. The mechanism of protection proposed by Brown and Gaugler (1998) was that glycerol removed body water osmotically from the nematodes, since the nematode cuticle is not permeable enough to allow the glycerol to enter the body to reduce cell membrane damage. There is no glycerol inside the infected host, however, there are significant stores of several different polyols (for a detailed description, see Abu Hatab and Gaugler, 2001). Indeed, sugars and polyols often play a role in cryoprotection of nematodes (Glazer, 2002). *G. mellonella* larvae contain approximately 70% lipid while they are alive (Abu Hatab and Shapiro-Ilan, unpublished data). It is possible that the lipids contained in the host act in a similar way to glycerol, and limit the damage caused to nematodes by disruption of membranes during freezing.

Our results and others also suggest that entomopathogenic nematodes may enjoy enhanced survival during harsh conditions within their cadavers. When IJs were frozen in sand for 1 week or less, they experienced significant levels of mortality. *Heterorhabditis bacteriophora* in particular showed improved survival inside the cadaver since there was no survival after 7 days at −8 °C when frozen as IJs in sand, yet when cadavers were frozen at the same temperature for 7 days, there was not even a significant decrease in IJ production. Brown and Gaugler (1996) found that exposure of cadavers with entomopathogenic nematodes to conditions of cold temperatures and low humidity delayed emergence, thereby enhancing IJ survival in desiccating conditions. Their rationale was that the IJs that remained inside the cadaver had higher rates of survival than those outside the cadaver in the same conditions. Koppenhofer et al. (1997) also suggested that entomopathogenic nematodes remain inside their host cadaver during dry soil conditions, which resulted in extended survival during harsh environmental conditions. In natural infections, all of these studies suggest that the cadaver might play a significant role in entomopathogenic nematode survival during adverse conditions in the field.

Can the cadaver's role in survival be exploited for biological control? There are significant advantages to applying entomopathogenic nematodes as cadavers, not the least of which is that this more closely reflects the conditions that occur in natural entomopathogenic nematode populations. We have shown that *H. bacteriophora* survival of freezing temperatures inside the cadaver is better than their IJ survival outside the cadaver during freezing conditions. This species also has greater dispersal (Shapiro and Glazer, 1996) and higher levels of infectivity (Shapiro and Lewis, 1999) when they emerge directly from their cadaver into a soil medium than when they are collected in water and subsequently applied to the same medium. Brown and Gaugler (1996) and Koppenhofer et al. (1997) also showed that entomopathogenic nematodes survive desiccation and freezing better inside their cadavers. Shapiro-Ilan et al. (2001) have tested a number of methods of formulating *H. bacteriophora*-infected insects with the goal of developing a usable entomopathogenic nematode formulation based upon these cadavers. It is conceivable that freezing may be used to store entomopathogenic nematode infected cadavers prior to application. The nematode, *H. bacteriophora*, appears to hold the most promise in this approach.

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